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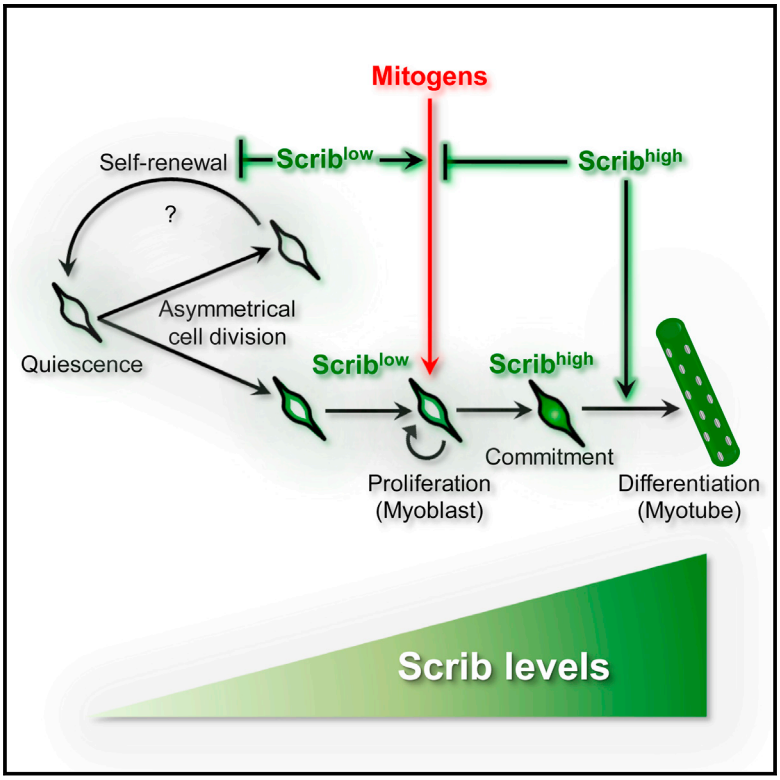
Cell Reports

Muscle Stem Cell Fate Is Controlled by the Cell-Polarity Protein Scrib

Graphical Abstract



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In Brief

The role of the cell-polarity protein Scrib in tissue stem cells is unclear. Ono et al. show that Scrib is a regulator of myogenic progression, controlling population expansion and self-renewal with expression levels of Scrib in muscle stem cells.

Highlights

- Scrib is asymmetrically distributed in dividing satellite cells
- Satellite cell fate is dictated by the level of Scrib
- Scrib mediates growth factor signaling in activated satellite cells
- Scrib is indispensable for muscle regeneration in vivo

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Muscle Stem Cell Fate Is Controlled by the Cell-Polarity Protein Scrib

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SUMMARY

Satellite cells are resident skeletal muscle stem cells that supply myonuclei for homeostasis, hypertrophy, and repair in adult muscle. Scrib is one of the major cell-polarity proteins, acting as a potent tumor suppressor in epithelial cells. Here, we show that Scrib also controls satellite-cell-fate decisions in adult mice. Scrib is undetectable in quiescent cells but becomes expressed during activation. Scrib is asymmetrically distributed in dividing daughter cells, with robust accumulation in cells committed to myogenic differentiation. Low Scrib expression is associated with the proliferative state and preventing self-renewal, whereas high Scrib levels reduce satellite cell proliferation. Satellite-cell-specific knockout of Scrib in mice causes a drastic and insurmountable defect in muscle regeneration. Thus, Scrib is a regulator of tissue stem cells, controlling population expansion and self-renewal with Scrib expression dynamics directing satellite cell fate.

INTRODUCTION

Muscle satellite cells, the resident tissue stem cells of skeletal muscle, provide myonuclei for postnatal muscle growth and for maintenance, repair/regeneration, and hypertrophy in adults (Montarras et al., 2013; Relaix and Zammit, 2012; Yin et al., 2013). Satellite cells are mitotically quiescent in healthy adult muscle but are activated in response to stimulation, such as muscle injury, to become myoblasts and proliferate extensively. The majority of satellite cell progeny then undergo myogenic differentiation to produce new myonuclei, whereas others return to a quiescent state to self-renew and replenish the stem cell pool. Understanding how satellite cell fates of differentiation or self-renewal are regulated is a central question in muscle biology and regenerative medicine and is of particular relevance to the failure of muscle maintenance and repair in muscle diseases such as Duchenne muscular dystrophy and age-related sarcopenia (Brack and Rando, 2012; García-Prat et al., 2013).

Accumulating evidence shows that cell polarity is important for a variety of cell functions, including migration and morphogenesis during tissue development, as well as adult tissue regeneration. Conversely, lack of cell polarization can lead to tissue disorganization and result in disease, including cancer (Martin-Belmonte and Perez-Moreno, 2012). Epithelial cells are highly polarized with apical and basolateral membrane compartments. This cell polarity is organized by three evolutionary conserved cell-polarity protein complexes: the apical partitioning defective (PAR), Crumbs, and the basolateral Scribble (Scrib) complexes. The Scrib polarity complex is composed of Dlg1-4, Lgl1/2, and Scrib, localized to the basolateral compartment. Scrib is a scaffold protein, containing 16 leucine-rich repeats and four PDZ (PSD-95/Dlg/ZO-1) domains. It has been identified and characterized as a neoplastic tumor suppressor in the epithelia of *Drosophila melanogaster* and has recently begun to be characterized in mammals as a multifunctional protein involved in regulation of cell proliferation, migration, apoptosis, and adherence, in addition to its role in cell polarity. Scrib is often mislocalized and universally overexpressed in various types of tumors, such as colon, breast, lung, ovary, and prostate cancers in humans (Vaira et al., 2011). Furthermore, loss of Scrib function causes disruption of apical-basal polarity and junctional integrity and inappropriate proliferation, resulting in tissue overgrowth (Martin-Belmonte and Perez-Moreno, 2012). Importantly, Scrib-heterozygous mice exhibit prostate hyperplasia, and conditional Scrib deletion in the prostate epithelium promotes neoplastic tumor progression through upregulation of the mitogen-activated protein kinase cascade that normally acts to accelerate tumorigenesis (Pearson et al., 2011). Although Scrib clearly plays a crucial role in preventing cancer progression in both

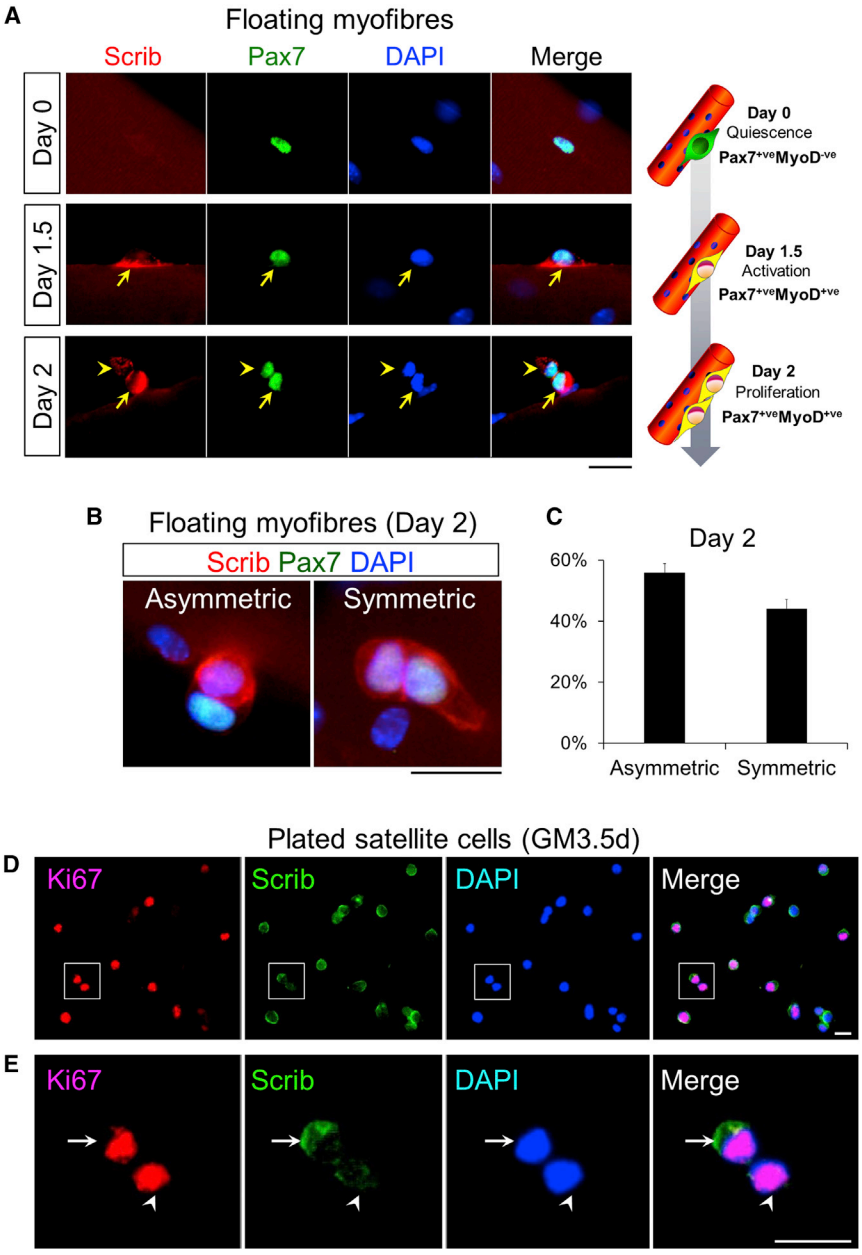

Figure 1. Scrib Is Upregulated during Satellite Cell Activation and Asymmetrically and Symmetrically Polarized in Dividing Cells

(A) Isolated EDL myofibers with their associated satellite cells were either immediately fixed (day 0) or cultured in plating medium (PM) for either 1.5 days (day 1.5) or 2 days (day 2) before fixation and immunostaining for Scrib and Pax7. On day 2, asymmetric distribution of Scrib was observed (arrow, high accumulation; arrowhead, low accumulation) during the first division of satellite cells.

(B and C) Satellite cells in their niche on isolated myofibers were cultured for 2 days. Scrib was distributed both asymmetrically and symmetrically in dividing satellite cell pairs (quantified in C; n = 3 mice, >15 pairs per mouse). Asymmetric or symmetric segregation of Scrib was defined based on the measured level of immunosignal intensity (a cell in an asymmetric pair must have at least double the fluorescence intensity of the other).

(D and E) Immunocytochemistry on plated satellite cells confirmed that the asymmetric distribution of Scrib protein was also observed in dividing cells directly plated on Matrigel and maintained in growth medium (GM) for 3.5 days after isolation (magnified in E).

Data represent means ± SEM. Representative data from at least three individual mice are shown. Scale bar represents 20 μm.

RESULTS

Scrib Is Expressed in Activated Satellite Cells and Becomes Asymmetrically and Symmetrically Polarized in Dividing Cells

Quiescent satellite cells express Pax7 in adult muscle. Upon stimulation, activated satellite cells upregulate the myogenic regulatory factor MyoD and then proliferate. Following population expansion, most satellite cells downregulate Pax7, maintain MyoD, express myogenin (Myog), and undergo myogenic differentiation (Halevy et al., 2004; Zammit et al., 2004).

To first examine the expression profile of Scrib during myogenic progression, we immunostained murine satellite cells retained in their niche on myofibers isolated from the extensor digitorum longus (EDL) muscle, as previously described (Ono et al., 2012). Immunofluorescence showed that Pax7^{+ve} quiescent satellite cells on freshly isolated myofibers (day 0) did not express Scrib (Figure 1A). However, after culturing in mitogen-rich medium (termed plating medium [PM]) for 1.5 (day 1.5) or 2 days (day 2), Scrib became expressed in activated and proliferating satellite cells (Figure 1A). During the first cell division, Scrib had an asymmetric distribution in 55.9% ± 2.9% of pairs of daughter cells, being at a higher level in one cell compared to the other, but exhibited a symmetric accumulation in others (44.1% ± 2.9%) (Figures 1B and 1C). At day 2 in non-adherent myofiber culture, we also confirmed that

invertebrates and vertebrates, whether or not Scrib also has a function in tissue stem cells remains unclear (Hawkins et al., 2013).

Cell-polarity proteins have also been proposed to act as potential regulators of asymmetric cell division, allowing a stem cell to generate a daughter cell that self-renews and another that undergoes differentiation (Conboy and Rando, 2002; Kuang et al., 2007; Shinin et al., 2006; Troy et al., 2012). This is in part controlled by non-canonical Wnt signaling (Le Grand et al., 2009). However, little is known about the role of polarity proteins in satellite-cell-fate choice during myogenic progression. Here, we investigated whether Scrib is involved in satellite-cell-fate decisions.

73.8% ± 3.0% of single Pax7^{+ve} cells exhibited a low expression of Scrib, while 26.2% ± 3.0% highly expressed Scrib (data not shown), consistent with the proportion of asymmetric cell divisions (Figure 1C). Next, to determine whether asymmetric distribution of Scrib protein could also be observed outside of the satellite cell niche, we plated satellite cells in adherent culture conditions and stimulated them with growth medium (GM) for 3.5 days. Immunostaining showed that Scrib protein remained polarized in dividing cells (Figures 1D and 1E), as observed with satellite cells in their niche (Figure 1B).

Scrib Is Upregulated in Cells Committed to Myogenic Differentiation, but Low/Negative Expression of Scrib Is Observed in Self-Renewing Cells

To examine the relationship between the level of Scrib and satellite cell fate, we co-immunostained adherent satellite cells for Scrib and cell-fate markers. In our culture model at day 7 in GM after isolation, the majority of cells express markers such as Pax7 and remain proliferative, indicative of undifferentiated cells, with only 5%–10% beginning to undergo myogenic differentiation as shown by Myog expression (data not shown). All cells with low Scrib levels (Scrib^{low}) were Pax7^{+ve}Myog^{-ve}, whereas all cells with high Scrib expression (Scrib^{high}) were Pax7^{-ve}, Ki67^{-ve} but Myog^{+ve} (Figure 2A, quantified in Figure 2B). A population with intermediate levels of Scrib expression (Scrib^{intermediate}) could express Pax7 or Myog, so exhibited a mixed profile, unlike Scrib^{high} and Scrib^{low} populations (Figures 2A and 2B). qPCR analysis showed that expression of *Scrib* is highly upregulated during myogenic differentiation induced by serum-reduced medium (differentiation medium [DM]) for 3 days (Figure 2C).

On stimulation by PM for 3 days in non-adherent myofiber culture, the Pax7^{+ve}MyoD^{+ve} activated/proliferative (Ki67^{+ve}) satellite cells undergo different fates: Pax7^{+ve}MyoD^{-ve} cells likely self-renew to return to a quiescent-like state, while Pax7^{-ve}MyoD^{+ve} cells are expressing Myog and committing to differentiation (Figure 2D) (Ono et al., 2011; Zammit et al., 2004). In satellite cells cultured for 3 days while retained in their niche on a myofiber, the same association between Scrib levels and fate was evident: all Scrib^{high} cells were Pax7^{-ve}MyoD^{+ve} and Myog^{+ve} cells committed to myogenic differentiation, while Scrib$^{low/negative}$ cells were Ki67^{+ve} proliferating or Pax7^{+ve}MyoD^{-ve} self-renewing cells (Figure 2D). We also confirmed that expression of Scrib was markedly lower in cells with the Pax7^{+ve} and MyoD^{-ve} self-renewal phenotype, but robustly upregulated in differentiating myotubes, under adherence culture conditions (Figure 2E). Thus, these data indicate that Scrib protein becomes expressed in activated cells and can be distributed to daughter cells, with high accumulation in cells committed to myogenic differentiation and lower levels in proliferating or self-renewing cells (Figure 2F).

In epithelial cells, Scrib protein levels are controlled by HSP90-mediated stabilization (Eastburn et al., 2012). To determine if such a mechanism also operates in satellite cells to stabilize Scrib, satellite cells were isolated from wild-type mice and cultured in GM (Figure 2A). Immunoblotting analysis demonstrated that treatment with the HSP90 inhibitor 17-AAG, at 1 μM for 24 hr, caused downregulation of Scrib protein compared with controls (Figure S1).

Scrib Is Indispensable for Muscle Regeneration In Vivo

Scrib-deficient (*Scrib*$^{-/-}$) mice are embryonic lethal (Murdoch et al., 2003; Pearson et al., 2011), so it is impossible to examine the function of Scrib in adults. To investigate the effects of loss of function of Scrib in satellite cells in adult mice, we generated satellite-cell-specific conditional Scrib-knockout mice by crossing *Pax7*$^{CreERT2/+}$ mice (Lepper and Fan, 2010) with Scrib-floxed (*Scrib*$^{f/f}$) mice (Pearson et al., 2011). Genetic inactivation of Scrib was induced by repeated intraperitoneal injection of tamoxifen (TMX) in *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$ mice (termed here *Scrib-scKO*), with TMX-treated *Scrib*$^{f/f}$ mice used as a control.

To first evaluate the role of Scrib in muscle regeneration in vivo, we genetically inactivated *Scrib* by five daily intraperitoneal injections of TMX in *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$, followed 5 days later by an intramuscular injection of cardiotoxin (CTX) to induce regeneration in the tibialis anterior (TA) muscle (Figure 3A). Regenerating TA muscles were removed 3.5 days after CTX injection and cryosectioned to study the effects of the loss of Scrib at the early stages of muscle regeneration, when numerous proliferative and differentiating cells are normally present (Ono et al., 2009). We first co-immunostained for Ki67 and MyoD to identify proliferating satellite-cell-derived myoblasts and found that Scrib-inactivation resulted in a significant decrease in the number of Ki67^{+ve} proliferating and Ki67^{-ve} non-proliferating myoblasts (Figures 3B and 3C). To quantify myogenic differentiation, sections were co-immunostained for laminin and developmental myosin heavy chain (dMyHC), which is transiently expressed in newly formed/immature myofibers. TMX-treated *Scrib-scKO* mice had a remarkable decline in the expression of dMyHC compared with control mice (Figures 3D and 3E). Thus, these data indicate that Scrib in satellite cells plays an important role in ensuring sufficient myoblasts are available for timely differentiation during regeneration after muscle injury.

To determine whether the lack of Scrib in satellite cells merely delays regeneration or actually blocks it, we examined regeneration 14 days after CTX injection (CTX14d), a time point when regenerating muscles have largely recovered in wild-type mice. Genetic inactivation of Scrib in satellite cells led to a marked decrease in muscle weight, down to ~40% of control levels at CTX14d (Figure 3F). Regenerating muscle cryosections were co-immunostained either for myosin heavy chain (MyHC) and laminin to analyze the cross-sectional area of myofibers (CSA) or for MyHC and collagen type I to assess fibrosis. The CSAs of regenerated myofibers (centrally nucleated) in *Scrib-scKO* mice were significantly reduced compared to controls at CTX14d (Figure 3G, quantified in Figure 3H). Immunohistochemistry also revealed that *Scrib-scKO* mice exhibited incomplete regeneration with marked fibrosis at CTX14d (Figure 3G, quantified in Figure 3I). Taken together, our data show that Scrib function in satellite cells is indispensable for muscle regeneration in vivo, likely affecting efficient satellite cell population expansion for timely myogenic differentiation during regeneration.

Loss of Scrib Impairs Population Expansion of Activated Satellite Cells

Given that population expansion in Scrib inactivated satellite cells was markedly decreased in vivo (Figure 3), we next focused on how the proliferative state of satellite cells is affected by loss of

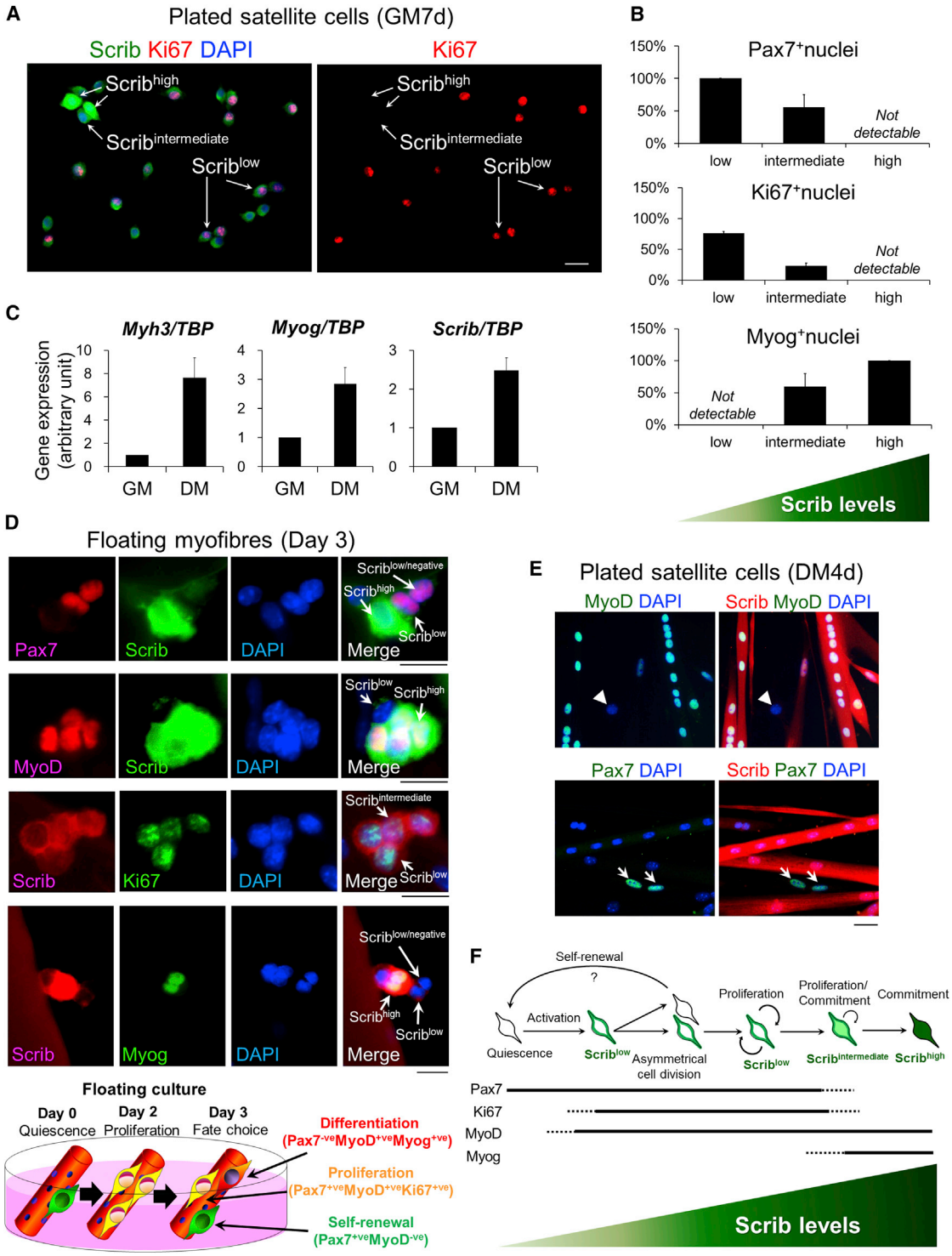

Figure 2. Scrib Protein Is Expressed in a Stage-Specific Manner during Myogenic Progression

(A and B) Plated satellite cells were cultured in GM for 7 days after isolation and co-immunostained for Scrib with Pax7, Ki67, or Myog.

(A) Expression levels of Scrib protein varied in plated satellite cells. Based on the immunofluorescence intensity for Scrib, cells were assigned to one of three categories: Scrib^{high}, Scrib^{intermediate}, and Scrib^{low}.

(B) All Scrib^{low} cells were undifferentiated Pax7^{+ve}Myog^{-ve} and most were proliferative as shown by being Ki67^{+ve}. In contrast, all Scrib^{high} cells were Myog^{+ve} and thus committed to myogenic differentiation, consistent with also expressing neither Ki67 nor Pax7.

(C) qPCR demonstrated Scrib expression increased in plated satellite cells during differentiation after being maintained in GM for 7 days or DM for 3 days.

(legend continued on next page)

Scrib. Mice were treated for 5 days with TMX, and myofibers were isolated after the last injection and cultured for 2 days (Figure 4A). Consistent with the undetectable expression of Scrib in quiescent satellite cells (Figure 1), the numbers of quiescent satellite cells per EDL myofiber of *Scrib-scKO* mice were unchanged when examined 5 days after the last injection of the 5-day TMX-treatment regime (Figure 4B). Immunofluorescence analysis confirmed that Scrib protein was efficiently deleted in activated Pax7^{+ve} satellite cells associated with myofibers cultured in PM for 2 days (Figure 4C). We evaluated the effect of Scrib inactivation on the proliferation of satellite cells by plating myofibers in adherent culture conditions and stimulating with GM for 6 days. We found abundant proliferating satellite cells from myofibers isolated from control mice, whereas myofibers from *Scrib-scKO* were surrounded by few satellite cells (Figure 4D).

To determine whether this proliferation defect still occurred if Scrib was lost following several cell divisions, we isolated satellite cells from *Pax7$^{CreERT2/+}$;Scrib$^{f/f}$* mice, plated them in GM to stimulate proliferation for 3 days, and then induced Scrib inactivation with 4-hydroxy tamoxifen (4OH TMX) for 2 days (Figure 4E). The proportion of Ki67^{+ve} proliferating cells was significantly reduced in TMX-treated, *Scrib*-inactivated satellite cells compared to those not exposed to 4OH TMX (Figures 4E–4H). Moreover, we confirmed that reduced Scrib levels resulted in a decreased proportion of Ki67^{+ve} satellite cells in wild-type mice using small interfering RNA (siRNA)-mediated knockdown of Scrib (Figures 4I and 4J), thus producing the same phenotype as the genetic inactivation of Scrib (Figures 4E–4H). Thus, loss of function of Scrib protein leads to suppression of progenitor expansion in satellite cells, even though Scrib acts as a tumor suppressor in *Drosophila* and mammalian epithelial cells (Martin-Belmonte and Perez-Moreno, 2012; Nagasaka et al., 2006).

Our in vivo and in vitro experiments suggested that Scrib is required for satellite cell population expansion after activation (Figures 3 and 4). Thus, we next investigated the effect of Scrib inactivation on fate decisions in satellite cells in non-adherent myofiber cultures (Figures 5A–5F). The total number of satellite cells was reduced, affecting all categories (i.e., Pax7^{-ve}MyoD^{+ve} differentiation-committed cells, Pax7^{+ve}MyoD^{+ve} proliferating cells, and Pax7^{+ve}MyoD^{-ve} self-renewing cells) in *Scrib-scKO* mice compared to control (Figure 5B, quantified in Figure 5C). Similarly, the total number of satellite cells was also significantly reduced on myofibers from wild-type mice treated with siRNA to knock down Scrib, but only Pax7^{-ve}MyoD^{+ve} committed cells and Pax7^{+ve}MyoD^{-ve} self-renewing cells were affected, with the proportion of Pax7^{+ve}MyoD^{+ve} activated or proliferating satellite cells unchanged (Figures 5D–5F).

Satellite Cell Fate Is Dictated by the Level of Scrib

Having shown that Scrib inactivation leads to defective progenitor expansion in satellite cells, we next examined the effects of constitutive expression of Scrib. We constructed a retroviral expression vector that encodes full-length human SCRIB, together with EGFP from an *IRES* to identify infected cells: *pMSCV-SCRIB-IRES-EGFP* (*SCRIB-RV*). Infection with the retroviral backbone *pMSCV-IRES-EGFP* (*Cont-RV*) served as the control.

Plated satellite cell progeny from *Scrib-scKO* mice were exposed to either *Cont-RV* or *SCRIB-RV* and then treated with 4OH TMX for 3 days to genetically delete *Scrib* in satellite cells, leaving only ubiquitously RV-driven *SCRIB* expression (Figure 6A). Co-immunostaining for EGFP and Scrib confirmed that the most EGFP^{+ve} satellite cells infected with *SCRIB-RV* expressed SCRIB protein at a relatively higher level than non-infected (EGFP^{-ve}) cells (Figure 6B). As endogenous Scrib protein is markedly upregulated in differentiating cells in DM (Figure 2D), immunostaining did not show obviously enhanced Scrib levels from the *RV*-mediated exogenous SCRIB protein compared to the already high levels in uninfected cells (data not shown). As expected, the proportion of Ki67^{+ve} proliferating cells significantly declined in 4OH TMX-treated *Scrib*-null cells compared with cells not exposed to 4OH TMX. Importantly, proliferation was successfully rescued by *RV*-mediated exogenous *SCRIB* expression in the genetically modified *Scrib*-null background (Figures 6C and 6D).

Under culture conditions designed to stimulate myogenic differentiation, most cells differentiate, but others exit the cell cycle with downregulation of MyoD and maintenance of Pax7, entering a quiescent-like state (Ono et al., 2009; Zammit et al., 2004). Cell cultures expressing *RV*-encoded *SCRIB* with endogenous Scrib inactivated were switched to differentiation medium for 5 days. Mononucleated satellite cells co-immunostained for EGFP with either Pax7 or MyoD revealed that constitutively expressed *SCRIB* drastically reduced MyoD^{-ve} cells but did not influence the proportion of Pax7^{+ve} cells. Since MyoD failed to be downregulated in the presence of SCRIB following induction of myogenic differentiation for 5 days (Figures 6E–6G), this indicates that reduction of Scrib is necessary to undergo self-renewal in satellite cells.

We next tested the effect of high SCRIB expression on myogenic progression. To achieve high overexpression of SCRIB, we transfected a plasmid encoding the full-length human *SCRIB* gene driven by a *CMV* promoter (*pCMV-SCRIB*) into satellite-cell-derived myoblasts. Immunostaining revealed that overexpression of SCRIB markedly suppressed the proportion of both Ki67^{+ve} proliferative cells in GM and Pax7^{+ve} undifferentiated/self-renewing cells in DM (Figures 6H–6M), showing that Scrib levels influence satellite-cell-fate choice. We also found that high-SCRIB-overexpressing cells have accelerated myogenic differentiation when cultured in DM, as shown by the proportion of nuclei in myotubes (Figure S2), although overexpression of SCRIB did not induce Myog expression in

(D) Satellite cells in their EDL myofiber niche were cultured in PM for 3 days before fixation and co-immunostaining for Scrib with Pax7, MyoD, Myog, or Ki67. Pax7^{+ve} undifferentiated cells, Ki67^{+ve} proliferating cells, or MyoD^{-ve} self-renewing cells were also observed in Scrib^{low} cell populations, but not in Scrib^{high} cells.
(E) Immunocytochemistry for Scrib together with either Pax7 or MyoD showed that expression of Scrib was increased in differentiating myotubes and decreased in MyoD^{-ve} or Pax7^{+ve} self-renewing satellite cells maintained in DM for 4 days (DM4d) (arrowhead, MyoD^{-ve} nuclei; arrows, Pax7^{+ve} nuclei).
(F) Schematic of expression patterns of Scrib protein during myogenic progression in satellite cells.
Data represent means ± SEM. Representative data from at least three individual mice are shown. Scale bar represents 20 μm.

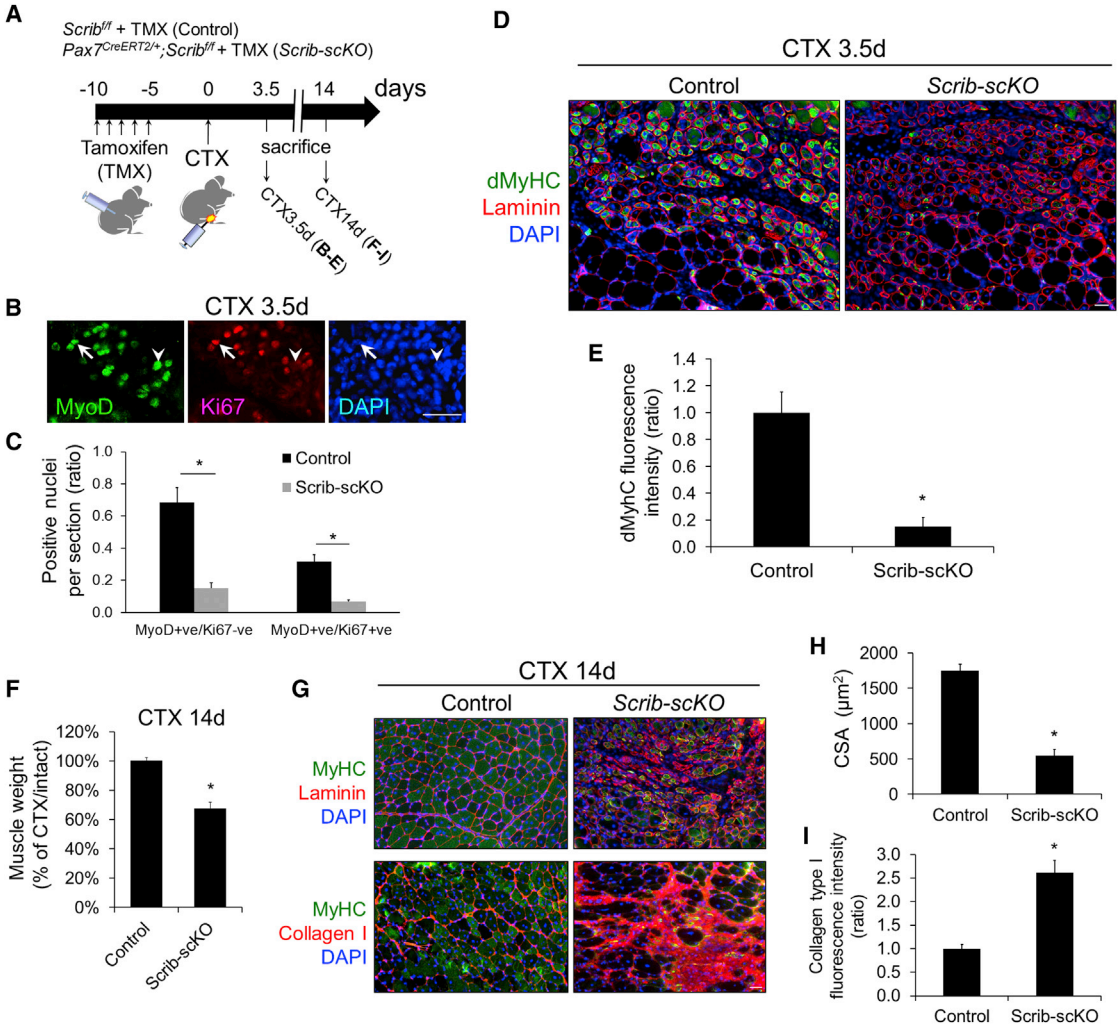

Figure 3. Scrib Is Critical for Muscle Regeneration In Vivo

To examine the role of Scrib in satellite cells in vivo, muscle regeneration was induced in TA muscles by injection of CTX in satellite-cell-specific Scrib-knockout mice.

(A) Schedule of TMX treatment before CTX injection and sacrifice of *Scrib*$^{f/f}$ (control) and *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$ (*Scrib-scKO*) mice.

(B–E) Mice were sacrificed at 3.5 days after CTX injection (CTX 3.5d) and assayed by immunostaining cryosections for either MyoD and Ki67 (B) or dMyHC and laminin (D; quantified in C and E, respectively [n = 3 mice, one to four cross-sectional fields (×10) of regenerating muscle per mouse were counted]). Knockout of Scrib in satellite cells resulted in a significant decrease in the number of both MyoD^{+ve}Ki67^{+ve} proliferating cells and dMyHC^{+ve} regenerating fiber compared with controls.

(F–I) Regenerated TA muscles from mice sacrificed 14 days following CTX injection had not reached control levels in *Scrib*-knockout mice, unlike controls (F; n = 5 mice). Immunostaining cryosections for either MyHC and laminin or MyHC and collagen type I (G) (quantified in H and I, respectively [n = 3 mice, >200 regenerating fibers per mouse were counted in H; n = 3 mice, more than three cross-sectional fields [×10] of regenerating muscle per mouse were counted in I) showed that Scrib inactivation in satellite cells led to a significant decrease in the mean CSA of centrally nucleated regenerating myofibers, compared to controls (H). Quantifying the degree of endomysial fibrosis using the collagen type I immunosignal revealed a marked increase in the fibrotic index in *Scrib-scKO* mice compared with controls (I).

Data represent means ± SEM. An asterisk denotes a significant difference from control (*$p < 0.05$). Representative data from at least three individual mice are shown. Scale bar represents 50 μm.

mitogen-rich growth medium (data not shown). We also found that reducing Scrib levels using siRNA did not prevent myogenic differentiation (Figure S3). Thus, these findings show that high levels of Scrib promote myogenesis after commitment to differentiation, but Scrib is dispensable for initiating the myogenic differentiation program.

Scrib Mediates Growth Factor Signaling in Activated Satellite Cells

Despite the high-serum culture conditions, our data show that satellite cell proliferation is impaired in *Scrib-scKO* mice (Figure 4D). We confirmed that expression levels of several key molecules in transforming growth factor β (TGF-β) (pSmad2), BMP

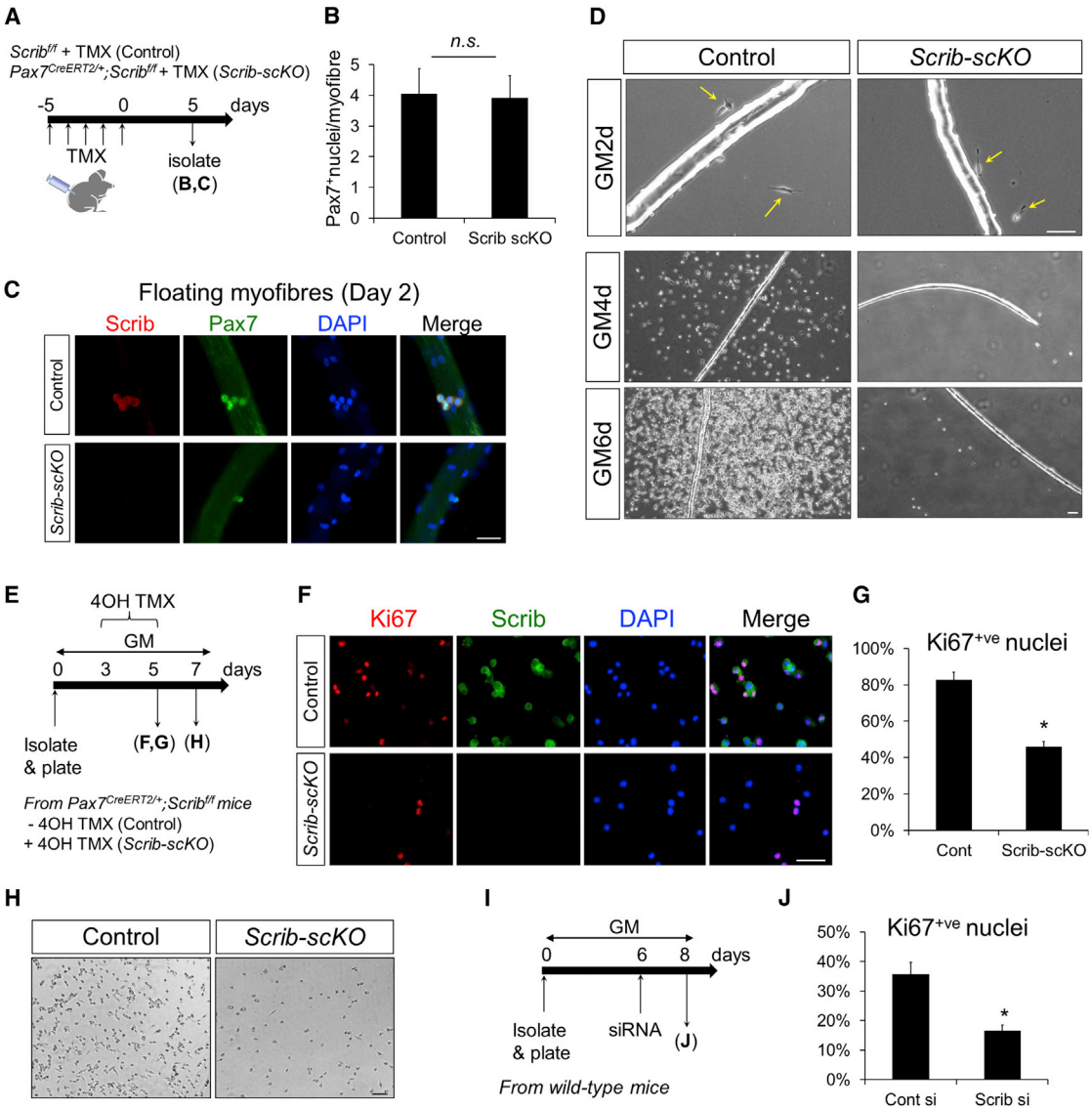

Figure 4. Scrib Deletion Impairs Proliferative Ability in Activated Satellite Cells

(A) Schedule of TMX injection to induce satellite-cell-specific conditional deletion for Scrib (*Scrib-scKO*) in *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$ mice. *Scrib*$^{f/f}$ mice were used as a control.

(B) Freshly isolated myofibers associated with satellite cells were fixed and immunostained for Pax7. There was no significant difference in the numbers of satellite cells between control and *Scrib-scKO* mice.

(C) Satellite cells associated with myofibers were isolated and cultured for 2 days in PM. Co-immunostaining for Scrib and Pax7 revealed that Scrib protein was efficiently deleted in *Scrib-scKO* mice.

(D) Satellite cells associated with myofibers were isolated and plated in the adherence culture model in GM for up to 6 days. The number of satellite cells that migrated from myofibers was clearly decreased in *Scrib-scKO* compared with control.

(E) Schedule of 4OH TMX treatment in plated satellite cells isolated from *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$ mice.

(F and G) Immunostaining for Ki67 and Scrib showed that the percentage of Ki67^{+ve} proliferative cells was decreased in Scrib-scKO satellite cells (quantified in G).

(H) Representative bright-field images show the lower cell density of *Scrib-scKO* cells compared with control. Satellite-cell-derived myoblasts were maintained in GM for 6 days.

(I) Time-course scheme of siRNA-mediated knockdown for Scrib in satellite cells isolated from wild-type mice.

(J) Immunostaining confirmed the percentage of Ki67^{+ve} cells was reduced in *Scrib*-knockdown cells.

In (B), 20 individual myofibers per mouse were counted (control, n = 5 mice; *Scrib-scKO*, n = 4 mice). Representative images from 20 individual myofibers from each of three mice (n = 3 mice) are shown in (C) and (D). More than 300 nuclei (G) or 100 nuclei (J) per mouse (n = 3–5 mice) were counted. Data represent means ± SEM. An asterisk denotes a significant difference from control (*$p < 0.05$). Scale bar represents 50 μm.

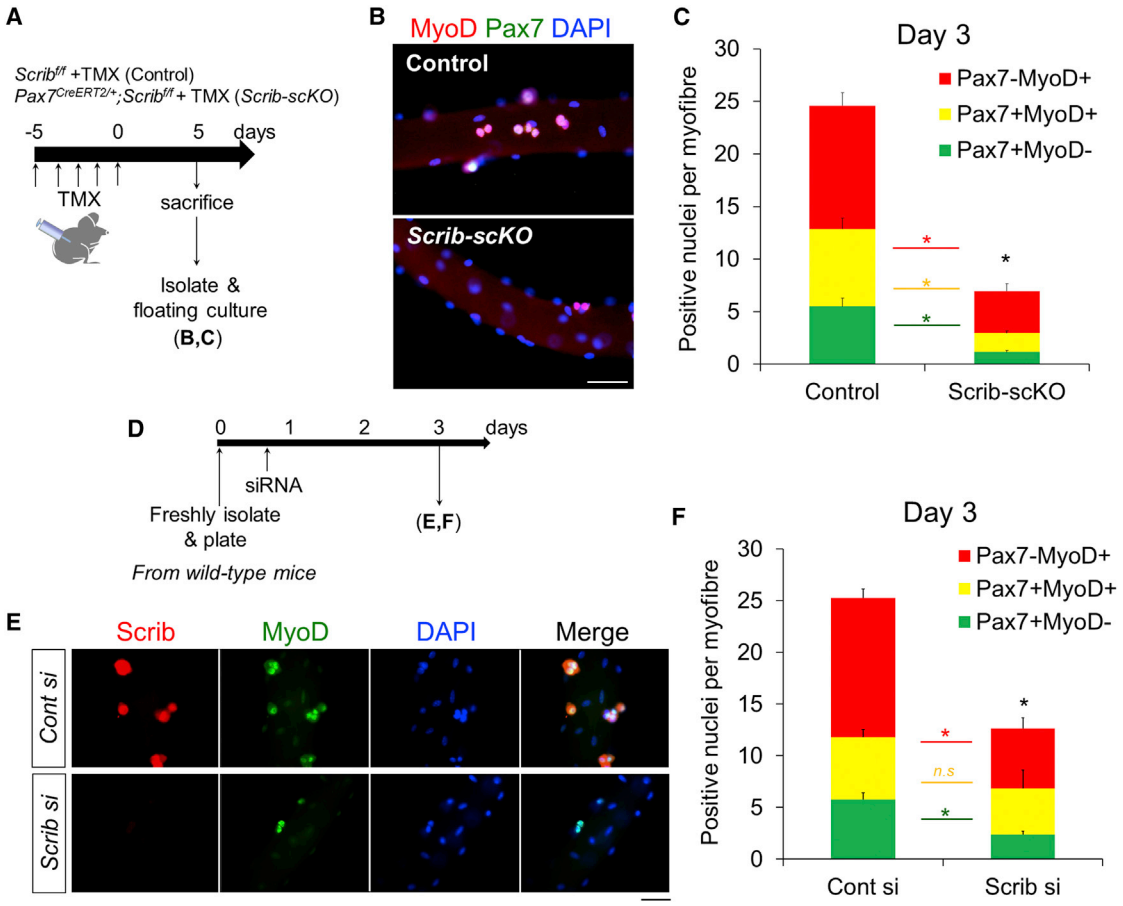

Figure 5. Scrib Inactivation Reduces Progenitor Population Expansion in the Niche

(A) Scrib inactivation in satellite cells retained in their niche was induced by TMX injection in *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$ mice.

(B and C) Isolated myofibers with their associated satellite cells were cultured in PM for 3 days and then immunostained for Pax7 and MyoD (quantified in C). The total number of cells per myofiber for all three populations (MyoD^{+ve}Pax7^{-ve}, MyoD^{+ve}Pax7^{+ve}, and MyoD^{-ve}Pax7^{+ve} cells) was remarkably decreased in *Scrib-scKO* satellite cells.

(D) Schedule of siRNA-mediated knockdown of Scrib in satellite cells.

(E and F) Satellite cells on myofibers were transfected with siRNA in floating culture 13–15 hr following activation with PM and immunostained for either MyoD and Scrib or MyoD and Pax7 on day 3 after isolation.

(E) Scrib protein was efficiently knocked down in MyoD^{+ve} satellite-cell-derived myoblasts by siRNA.

(F) The total number of cells per myofiber, especially in MyoD^{+ve}Pax7^{-ve} and MyoD^{-ve}Pax7^{+ve} cell populations, was significantly reduced in Scrib-knockdown cells (n = 3–5 mice per condition; more than ten individual myofibers per mouse were counted in C and F).

Data represent means ± SEM. An asterisk denotes a significant difference from control (*p < 0.05). Scale bar represents 50 μm.

(pSmad1/5/8), canonical Wnt (β-catenin), and Notch (Hey1), signaling pathways that regulate satellite cell fate, were unchanged in *Scrib-scKO* satellite cells in vitro (Figure S4B). Thus, we next investigated whether Scrib is involved in growth factor signaling in activated satellite cells by examining downstream mechanisms involving well-characterized kinases (Elsum et al., 2012). Several studies have reported that Scrib functions as a potent negative regulator for phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Nagasaka et al., 2010; Pearson et al., 2011) and positively regulates phosphorylation of c-Jun (Zhan et al., 2008). Consistent with these reports, when we genetically inactivated Scrib in satellite cells, expression of p-ERK1/2 increased while expression of p-c-Jun decreased (Figure 7B).

To further determine whether Scrib mediates signaling pathways by mitogens, we tested the effect of treatment with either insulin-like growth factor-1 (IGF-1) or tumor necrosis factor α (TNF-α), both of which are known to stimulate proliferation of satellite cells (Allen and Boxhorn, 1989; Alter et al., 2008; Li, 2003) and operate through kinases including p38, c-Jun, ATF2, and ERK1/2. Western blotting showed that stimulation with either IGF-1 (100 ng/ml) or TNF-α (5 ng/ml) for 20 min in control satellite-cell-derived myoblasts upregulated their target molecules, such as p-p38 and p-c-Jun. However, *Scrib*-deficient satellite cells failed to respond to stimulation with either IGF-1 or TNF-α by characteristic phosphorylation of these kinases (Figure 7C). In contrast, expression of p-ERK1/2 induced by IGF-1 in control satellite cells was markedly increased in *Scrib-scKO* cells (Figure 7C).

Because Scrib regulates growth factor signaling, we tested the effects of IGF-1 or TNF-α on satellite cell fate when Scrib was inactivated. EDL myofibers were isolated from TMX-treated *Scrib-scKO* mice, and the associated satellite cells pre-activated in PM for 1.5 days, before being exposed to IGF-1 (100 ng/ml) or TNF-α (5 ng/ml) for a further 1.5 days, then fixed and co-immunostained for MyoD and Pax7 (Figure 7D). Immunofluorescence analysis confirmed that treatment with IGF-1 or TNF-α promoted further population expansion of satellite cells from control mice, but their effects were negligible on cells from *Scrib-scKO* mice, with satellite cell fate generally unaffected (Figure 7E). Taken together, these data show that deregulation of growth factor signaling contributes to the defect in proliferative ability in *Scrib-scKO* satellite cells (see model in Figure 7F).

DISCUSSION

In this study, we investigated the role of Scrib, the cell-polarity protein in satellite cells during adult myogenesis. We found that quiescent cells do not express Scrib while activated satellite cells begin to express Scrib, which is then both asymmetrically and symmetrically distributed in dividing cells. Scrib protein tends to be divided into daughter cells with high accumulation/expression in cells undergoing myogenic differentiation and low accumulation/expression in proliferating or self-renewing cells. However, it remains unclear whether satellite cell progeny also upregulate the level of Scrib protein without asymmetric cell division or whether the asymmetric cell division is necessary to decide cell fate during myogenic progression.

Expression levels of Scrib also vary through myogenic progression. Thus, we characterized satellite cell fates based on levels of Scrib expression: an activated and proliferative Scrib^{low} population, a proliferative and committed Scrib^{intermediate} population, and a differentiating Scrib^{high} population (see model in Figure 7F).

Scrib is a well-known tumor suppressor, and loss of Scrib results in dysregulation of apical-basal polarity, leading to the formation of neoplastic tumors in epithelial tissue (Pearson et al., 2011; Zhan et al., 2008). However, the physiological function of Scrib in stem cells for tissue repair and regeneration was unknown. We found that loss of Scrib results in an impairment of progenitor population expansion in satellite cells. These observations were surprising, because Scrib possesses potent growth inhibitory activity in epithelial cells of *Drosophila* and mammals, indicating that Scrib has a distinct role in epithelial cells compared to tissue stem cells. Crucially, we showed that satellite-cell-specific genetic knockout of *Scrib* leads to smaller regenerated fibers and accumulation of fibrotic tissues after CTX-induced injury, concomitant with a significant decrease in the number of both proliferating and differentiating myogenic cells. Indeed, our data suggest that Scrib permits activated satellite cells to expand the progenitor population for appropriate muscle regeneration.

A recent study has revealed that overexpression of Scrib prevents cell-cycle progression by downregulation of Cyclin D1 in epithelial cells (Nagasaka et al., 2006). Consistent with this observation, we confirmed that *pCMV*-driven overexpression of Scrib downregulated Cyclin D1 (data not shown) and inhibited proliferation in satellite cell progeny. Interestingly, *RV*-mediated constitutive expression of Scrib did not influence proliferative ability. These data indicate that appropriate low-level Scrib expression (Scrib^{low}) maintains the proliferative state of activated satellite cells, whereas the robust accumulation of Scrib (Scrib^{high}) attenuates proliferation and promotes myogenesis. Importantly, both overexpression and constitutive expression of Scrib inhibit satellite cell self-renewal, suggesting that downregulation of Scrib is a necessary step to self-renew and become quiescent. Taken together, our data indicate that satellite-cell-fate decisions depend on levels of Scrib.

Scrib appears to act as a signaling scaffold, interacting with various signal-transduction molecules, such as ERK1/2, p38, and c-Jun N-terminal kinase (JNK)-Jun (Martin-Belmonte and Perez-Moreno, 2012; Norman et al., 2012). In *Drosophila*, Scrib controls apoptosis in a TNF-JNK-dependent mechanism (Igaki et al., 2009). A recent study has reported that Scrib maintains the level of p-c-Jun expression, stimulating a JNK-Jun-Bcl-2 pathway to induce cell death of tumor cells, thus preventing tumorigenesis (Zhan et al., 2008). In accord with these findings, we showed that genetic inactivation of Scrib in proliferative culture conditions resulted in an increase in expression of p-ERK1/2 as well as a decrease in expression of p-c-Jun. Recent studies have reported that p-ERK1/2 regulates satellite self-renewal (Abou-Khalil et al., 2009; Le Grand et al., 2012; Shea et al., 2010) and p-c-Jun sustains the proliferative state (Alter et al., 2008). Thus, we speculate that a lower level of p-c-Jun may be involved in the defective proliferation of *Scrib*-deficient satellite cell progeny. In addition, our preliminary data show that high levels of Scrib protein reduce the level of p-ERK1/2 (data not shown), indicating that self-renewal of satellite cells might be prevented, in part, through ERK1/2 inactivation by Scrib protein. ATF-2 and p38, including c-Jun, are described as part of the TNF-α or IGF-1 signaling pathways that can mediate population expansion of activated satellite cell progeny. Knockout of IGF or its receptor genes leads to a severe growth defect (Liu et al., 1993), and treatment with IGF-1 facilitates proliferation in primary cultured myoblasts (Allen and Boxhorn, 1989). Stimulation with TNF-α accelerates proliferation of primary myoblasts (Li, 2003), and therefore, TNF-α signaling has a preferential role in muscle regeneration after traumatic freezing injury or CTX-induced damage in vivo, shown using TNF receptor 1 and 2 double knockout or TNF-α antibody neutralization (Chen et al., 2005; Warren et al., 2002). Consistent with these findings, we show that treatment with either IGF-1 or TNF-α resulted in promotion of population expansion in Scrib$^{f/f}$ control satellite cells. Importantly, however, *Scrib*-deficient satellite cell progeny were less responsive to stimulation by these growth factors. Thus, Scrib may function in maintenance of progenitor expansion in activated satellite cells, at least in part, by modulating IGF-1 or TNF-α signaling pathway. We also showed that the level of protein kinase C iota, a part of PAR cell-polarity complex that regulates satellite cell fate (Troy et al., 2012), was not changed by reduced Scrib expression in satellite cells (Figure S4B). However, it remains unclear whether Scrib function is associated with other cell-polarity proteins and non-canonical Wnt signaling (Le Grand et al., 2009).

In conclusion, we have described the role of the cell-polarity protein Scrib, a crucial regulator of myogenic progression

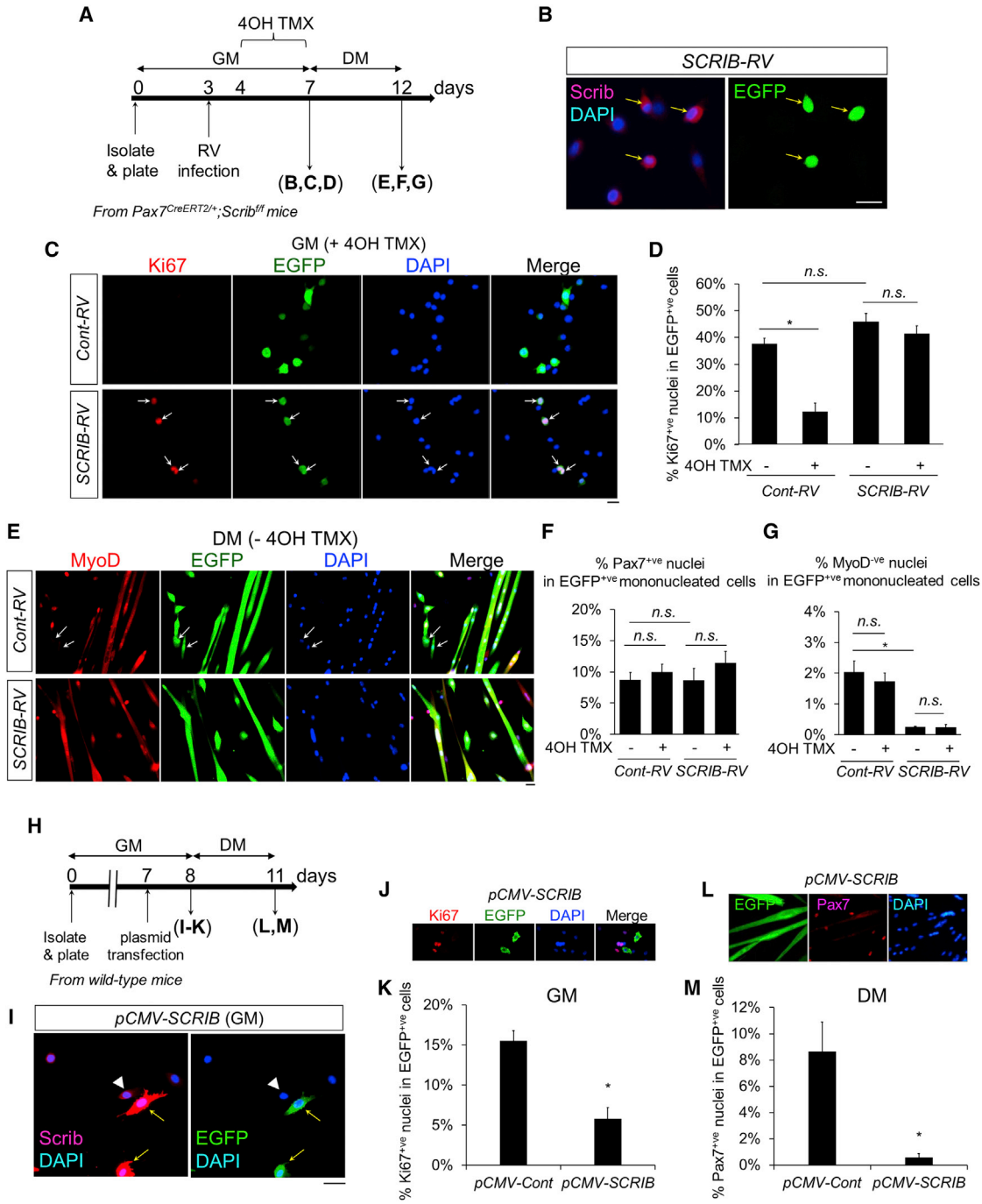

Figure 6. Level of Scrib Expression Influences Satellite Cell Fate

The effects of constitutively expressed or overexpressed Scrib on satellite-cell-fate decisions were analyzed.

(A) Schedule of 4OH TMX treatment and retrovirus infection in plated satellite cells isolated from *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$ mice. *pMSCV-SCRIB-IRES-EGFP* (*SCRIB-RV*) encoding full-length human *SCRIB* and *pMSCV-IRES-EGFP* (*Cont-RV*) were used for SCIRB-constitutive expression and control conditions, respectively.

(B) Typical images of satellite cells co-stained with Scrib and EGFP antibodies after *SCRIB-RV* infection. Arrows show *SCRIB-RV* infected EGFP^{+ve} satellite cells, expressing Scrib at a relatively higher level than non-infected cells.

(C and D) Plated satellite-cell-derived myoblasts were exposed to either *Cont-RV* or *SCRIB-RV* before cells were treated with 4OH TMX in GM for 3 days and co-immunostained for Ki67 and EGFP (quantified in D). The percentage of Ki67^{+ve} proliferative cells significantly decreased compared to cells without 4OH TMX, and this decline was completely rescued by *SCRIB-RV* infection.

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controlling population expansion and self-renewal, with expression levels of Scrib regulating satellite cell fate (Figure 7F). The appropriate levels of Scrib may be crucial to balance population expansion and self-renewal, likely through the degree of ERK1/2 and c-Jun activation. Despite the fact that Scrib is a key polarity factor that prevents outgrowth of tumor cells in epithelial tissues, Scrib acts as a critical mediator in population expansion in activated satellite cells. These observations imply that Scrib is not likely to have a universal anti-proliferative activity, and the role of Scrib may vary in a cell-type- or tissue-specific manner. Since our findings provide new insight into the molecular mechanisms that govern stem cell function, this informs development of stem-cell-based therapies for muscle-wasting diseases such as muscular dystrophies and age-related sarcopenia as well as tumor biology.

EXPERIMENTAL PROCEDURES

Satellite Cell Isolation and Culture

EDL muscles were isolated and digested in type I collagenase, as previously described (Ono et al., 2012). Satellite cells were obtained from isolated myofibers by trypsinization in a 0.125% trypsin-EDTA solution for 10 min at 37°C with 5% CO_2. Satellite cells were cultured in GM (GlutaMax DMEM supplemented with 30% fetal bovine serum, 1% chick embryo extract, 10 ng/ml basic fibroblast growth factor, and 1% penicillin-streptomycin) at 37°C with 5% CO_2. Myogenic differentiation was induced in DM (GlutaMax DMEM supplemented with 5% horse serum and 1% penicillin-streptomycin) at 37°C with 5% CO_2. For floating culture, isolated myofibers were cultured in PM (GlutaMax DMEM supplemented with 10% horse serum, 0.5% chick embryo extract, and 1% penicillin-streptomycin) at 37°C with 5% CO_2. For cell culture, 4OH TMX was used at a concentration of 1 μM. Satellite cells were stimulated by recombinant IGF-1 or TNF-α protein for 20 min following pre-incubation in serum-free medium for 6 hr.

Animals

Animal experimentation was approved by the experimental animal care and use committee of Nagasaki University. *Scrib-floxed* mice (Pearson et al., 2011) were crossed with $Pax7^{CreERT2}$ mice (Lepper and Fan, 2010) to generate $Scrib^{f/f}$ and $Pax7^{creERT2/+};Scrib^{f/f}$ mice. All mice used in this study had a *C57BL6* genetic background and were between 8 and 16 weeks old, with age-matched littermate controls.

Muscle Regeneration

TMX dissolved in corn oil (at a concentration of 5 μl per g of 20 mg/ml) was injected intraperitoneally daily for 5 days. To induce muscle injury, 50 μl of 10-μM CTX (Sigma-Aldrich) was injected intramuscularly into the tibialis anterior muscle of anaesthetized mice using a 29G 1/2 insulin syringe. Regenerating muscles were isolated 3.5 and 14 days after CTX injection, immediately frozen in 2-methylbutane cooled in liquid nitrogen, and stored at −80°C before being cryosectioned. Transverse sections of muscle were cut using a cryostat and immunostained.

Retroviral Infection and Plasmid Vectors

The retroviral backbone *pMSCV-puro* (Clontech) was modified to replace the puromycin selection gene with an *IRES-EGFP* in order to create *pMSCV-IRES-EGFP*, which served as the control (Zammit et al., 2006). Human *SCRIB* cDNA was cloned into *pMSCV-IRES-EGFP* to produce *pMSCV-SCRIB-IRES-EGFP* (*Scrib-RV*), expressing *SCRIB* and *EGFP* as a reporter. Retroviruses were packaged into the Platinum-A (PLAT-A) Retroviral Packaging Cell Line (Cell Biolabs) according to the manufacturer's instructions. Retroviral infection was performed with the PLAT-A supernatant supplemented with 4 μg/ml polybrene and left at 37°C for 3 hr. *pCMV-EGFP-SCRIB* plasmid (Nagasaka et al., 2006) or empty *pCMV-EGFP* plasmid as a control was transfected using Lipofectamine LTX with Plus Reagents (Life Technologies) into satellite-cell-derived myoblasts at 50%–60% confluence in adherence condition.

Statistical Analysis

Significant differences were determined using the Student's t test, with $p < 0.05$ considered as statistically significant. All data are means ± SEM.

Additional Methods

Antibodies and reagents and experimental details for immunoblotting, immunostaining, transfection, and qPCR are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.01.045.

AUTHOR CONTRIBUTIONS

Y.O. designed and performed the experiments, analyzed the data, assembled the input data, and wrote the main manuscript. Y.U., S.G., and T.L. provided technical support. S.N. and P.O.H. developed the analytical tools and interpreted the data. P.S.Z. interpreted the data, assembled the input data, and wrote the manuscript. All authors discussed the results and implications and commented on the manuscript.

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(E–G) *Cont-RV*- or *SCRIB-RV*-infected satellite-cell-derived myoblasts were induced to undergo myogenic differentiation in DM for 5 days and co-immunostained for EGFP and either Pax7 or MyoD in satellite cells in the presence or absence of 4OH TMX (E, quantified in F or G, respectively). Constitutively expressed Scrib did not influence Pax7 expression but prevented downregulation of MyoD expression (arrows show $EGFP^{+ve}MyoD^{-ve}$ cells).

(H and I) To test high-level expression of Scrib protein in satellite cells, the plasmid encoding EGFP-tagged human full-length SCRIB gene driven by a *CMV* promoter was transfected into satellite-cell-derived myoblasts. (H) Schedule of plasmid vector transfection in plated satellite cells isolated from wild-type mice. *pCMV-SCRIB-EGFP* (*pCMV-SCRIB*) encoding EGFP-tagged full-length human SCRIB and *pCMV-EGFP* (*pCMV-Cont*) were used for Scrib-overexpression and control conditions, respectively. (I) Representative images of satellite cells co-immunostained for EGFP and Scrib after *pCMV-SCRIB* transfection. *pCMV-SCRIB*-transfected $EGFP^{+ve}$ satellite cells (arrows) strongly expressed Scrib compared with non-transfected cells (arrowhead).

(J and K) Immunostaining for EGFP and Ki67 showed that *pCMV-SCRIB* significantly reduced the proportion of both $Ki67^{+ve}$ proliferative cells in GM (quantified in K).

(L and M) *pCMV-Cont* or *pCMV-SCRIB* transfected satellite cells were induced to differentiation in DM for 3 days and co-immunostained for EGFP and Pax7 (quantified in M). Overexpressed SCRIB decreased the percentage of $Pax7^{+ve}$ undifferentiated or self-renewing cells.

Data represent means ± SEM. In (D), (F), and (G), >300 EGFP+ nuclei were counted per mouse; in (K) and (M), >150 $EGFP^{+ve}$ nuclei were counted per mouse (n = 3 mice per condition). Representative data from at least three individual mice are shown. An asterisk denotes a significant difference from control (*$p < 0.05$). Scale bar represents 20 μm.

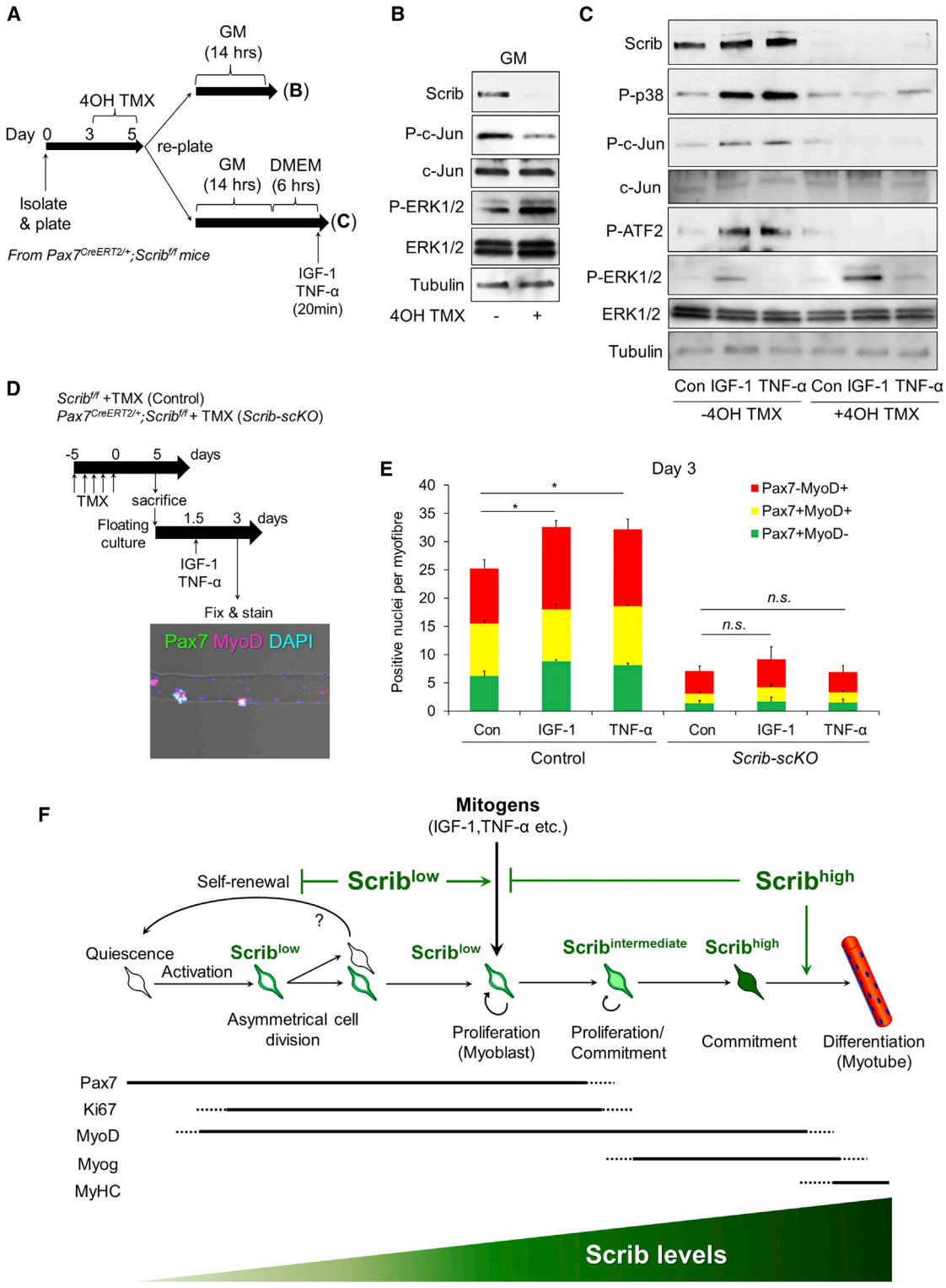

Figure 7. Scrib Is Required for the Response to Growth Factor Stimulation

(A) Experimental scheme of culture conditions for (B) and (C). Isolated satellite cells were cultured in the presence or absence of 4OH TMX in GM in *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$ mice.

(B) Immunoblotting analysis demonstrated that efficient knockout of Scrib decreased p-c-Jun and increased p-ERK1/2 protein levels in *Scrib-scKO* satellite cells compared with controls.

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(C) Satellite cells were stimulated by recombinant IGF-1 or TNF-α protein for 20 min, and immunoblot analysis was performed.
(D) Experimental schedule of culture conditions for myofibers. Scrib inactivation in satellite cells was induced by TMX injection in *Pax7*$^{CreERT2/+}$;*Scrib*$^{f/f}$ mice as described in Figure 5A.
(E) Isolated individual myofibers associated with satellite cells were activated in PM for 1.5 days and then treated with or without IGF-1 or TNF-α in PM for a further 1.5 days. Numbers of cells positive or negative for Pax7 and MyoD per myofiber were quantified (n = 3 mice per condition; more than 10 individual myofibers per mouse were counted). Data represent means ± SEM. An asterisk denotes a significant difference from control (*$p < 0.05$). Representative data from at least three individual mice are shown. Scale bar represents 20 μm.
(F) Role of Scrib during myogenic progression in satellite cells. During muscle repair and regeneration, quiescent satellite cells do not express Scrib but activated cells begin to express Scrib at low levels (Scrib^{low}). Scrib^{low} cells then proliferate extensively before the majority upregulate Scrib (Scrib^{high}) and commit to myogenic differentiation. Low-level Scrib expression operates through controlling signal pathways of mitogens, such as IGF-1 and TNF-α, in order to maintain the proliferative state and allow the myoblast population to expand, while preventing self-renewal. Conversely, high Scrib levels prevent proliferation of satellite cells to facilitate myogenic differentiation.

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